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These studies illustrate the versatility and potential usefulness of this bioluminescent reporter plasmid for studying the roles of EPS in biofilm process and the effects of other environmental insults on these microbial biofilms. Future experiments should allow the cellular processes involved in microbially induced corrosion to be successfully studied from a molecular point of view.

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Final Report **Date:** 01 January 1997

Contract #: N00014-93-1-1387

Principal Investigator: Drs. David C. White and Gary S. Sayler

Institute: Center for Environmental Biotechnology, The University of Tennessee, Knoxville.

Contract Title: Bioluminescent Genetically Engineered Microorganisms for the Study of Biofilms.

Period of Performance: 6 June 1993 through 31 May 1996

Objectives:

1. To develop bioluminescent reporter bacteria to study the effects of environmental stress factors on alginic acid biosynthesis.
2. To characterize these bacterial transconjugates in batch culture studies examining the effects of increasing culture osmolarity and decreases in nitrogen availability on exopolysaccharide production.
3. To use these bioluminescent transconjugates to study transcriptional regulation of alginate biosynthesis in developing biofilms.

Accomplishments:

1. Transconjugation of pUTK50 into environmental strains. Six environmental strains were chosen to be hosts for the bioreporter plasmid pUTK50 (Table 1), including *P. putida* L, P. *fluorescens* M, *P. fluorescens* N, *S. maltophilia O, P. syringae* P, and *P. aeruginosa* 322. These environmental bacteria were originally isolated from corroded pipes and reactor debris at a nuclear power plant and have demonstrated sequence homology to the regulatory and structural genes of the alginic acid biosynthetic pathway (*alg*D, *alg*G, *alg*-76, and *alg*B; Table 1). Five successful triparental transconjugations were performed with *P. putida* L, P. *fluorescens* M, *P. fluorescens* N, *S. maltophilia* O, *P. syringae* P. Unfortunately, attempts to mobilize pUTK50 into *P. aeruginosa* 322 were unsuccessful, because of an acquired resistance to both selection markers necessary for plasmid maintenance (kanamycin and carbenicillian).

2. Characterization of bioluminescent bacteria in batch cultures studies. In organisms which are known to produce copious amounts of alginate, such as *P. aeruginosa* FRD1, the high level of alginate produced was correlated with a high capacity for bioluminescent gene expression. It was also found that enhanced bioluminescence proceeded accumulation of *alg*D mRNA transcripts under conditions of NaCl induction, and that in induced cultures the abundance of *alg*D mRNA was prolonged relative to uninduced cultures (Figure 1 and 2). Possibly, this was due to prolonged stimulation of transcription from the *alg*D promoter, but it is also possible that the degradation rates for the mRNA were affected, which allowed accumulation of mRNA transcripts. The time of onset and rate of alginic acid accumulation in the culture medium did not reflect the degree of relative induction as seen in the bioluminescent light output, although alginic acid appeared to persist for a prolonged period of time in induced cultures. These data suggest that the rate of alginate synthesis was not greatly affected in this strain, but rather it was possible for alginate to be synthesized for an extended period of time.

Bioluminescent reporter strains, L23 and N15, demonstrated the lowest inducibility among the environmental bacteria (Table 2). L23 had the lowest overall bioluminescence among the environmental isolates, but this strain also had the highest MHB responses next to FRD1 (Table 3). This could be accounted for by remembering that in non-mucoid alginate producers the activity of the alginate biosynthetic enzymes is usually low. Therefore, it is probable that L23 can not become fully induced under these experimental conditions. N15 had the lowest alginate gene similarities but was the third

highest light producer in these experiments (1st FRD1, 2nd O46). Uronic acids were detected in N15 culture supernatants, but monosaccharides other than D-mannuronic and L-guluronic acid were observed by Dionex analysis (Figure 3). For this strain, it appears that the bioluminescent reporter plasmid is being induced by some undetermined factor in cell, which may be related to an alternative exopolymer synthesis pathway.

NaCl studies with M39 and O46 showed that these strains were very inducible, as indicated by the minimal bioluminescence of control cultures and the high bioluminescence in induced strains (Table 2). These bacterial strains were unreactive to MHB assays and ion chromatography did not detect significant quantities of the alginic acid constituents (Table 3). Additionally, monosaccharides not present in alginate were identified (Figure 3). It may be that, in these reporter strains, the bacteria have lost their ability to synthesis alginate at some point in the biosynthetic pathway, or that these bacteria have evolved another mechanism for control of an alternative exopolysaccharide, whose regulatory mechanism is somewhat homologous to the one found in alginate producers.

Mixtures of ammonium and nitrate ions, as a limited source of nitrogen, induced bioluminescence in the environmental isolates, L23 and N15, and there was a definite trend of decreasing nitrogen availability vs. increasing bioluminescence (Table 2). However, for the other environmental strains, M39 and O46, there was no significant trend in induction of bioluminescence in response to decreasing nitrogen availability, and although M39 and O46 exhibited a slight induction of the *alg*D promoter when grown in cultures containing 3.25mM NH_4^+/6.75mM NO_3^-, there was no induction in cultures containing only 10mM NO_3^- (Table 2). These data show that the bioluminescent responses vary among the environmental strains and do not necessarily correlate with the patterns of alginic acid synthesis observed in FRD1.

Exopolymers produced by the environments strains were isolated and characterized as being distinctly different from the bacterial alginates (Figure 3). These preliminary data do not discount the possibility that alginate can not be produced in the strains tested, but it appears that there is a predominance of alternative EPS within culture supernatants. It was observed that each of the bacterial strains produced a unique series of monosaccharides when hydrolyzed exopolymer was examined using ion chromatography. The biopolymer data seems to indicate that there may be more than one exopolysaccharide produced by the environmental bacteria. The complex composition of the supernatants from liquid culture studies could be explained by the accumulation of different EPS in these cultures.

3.Induction of alginic acid biosynthesis in developing biofilms using the bacterial transconjugates. The surface adherent environmental isolate, *S. maltophilia* O46, was chosen for studies using laminar flow biofilm reactors to monitor *in situ* light production and EPS synthesis within an actively growing biofilm. During initial experiments, O46 demonstrated a sustained ability for light production, under conditions necessary for biofilm growth and development (Figure 4). Additionally, this bacterium stabily maintained pUTK50 and allowed visualization of changes in light induction, throughout the course of the experiment. Exopolymer from O46 biofilms was found to contain D-glucose and one other unidentified sugar (Figure 5 and 6), but no uronic acids were identified in this exoploymer.

Subsequent experiments, using strain O46, examined the effects of an environmental stress factor on bioluminescence in an actively growing biofilm. It was shown, in these experiments, that bioluminescence was induced once biomass stabilized and that diminished bioluminescence preceded biofilm sloughing in control cultures (Figure 7). It was also shown that increases in NaCl concentrations resulted in reduced rates of biofilm formation and that bioluminescence was not induced in the initial stages of biofilm growth. NaCl stressed biofilms demonstrated abnormal growth in the early stages of the experiments and bioluminescence was shown to be induced in a secondary stage of biofilm growth, uniquely to the NaCl induced biofilms.

Significance:

The evidence presented in this research project does not correlate bioluminescence with alginic acid synthesis in environmental isolates L23, M39, N15, and O46. However, it does propose the possibility that alternate polysaccharides are produced and activators of their synthesis may also induce the *alg*D promoter. It appears that these exopolysaccharides could be regulated, transcriptionally, in a manner resembling traditional global response systems, such as is observed in other cellular responses such as

starvation and nitrogen deprivation. In fact, there is increasing evidence that alginate is regulated in response to changes in the bacteria's energy status and environmental stimuli previously shown to induce global cellular changes such as the heat shock response. In previous studies, the existence of alternate exopolysaccharides has been demonstrated in members of the *Pseudomonas* RNA homology group B sub-family, but this study is the first to suggest the possibility that these alternate exopolysaccharides may be controlled by genes which show some homology to the ones responsible for controlling the alginate biosynthetic pathway.

These studies demonstrated that specific periods of EPS induction seem to be important in biofilm processes. In the case of the control biofilms, exopolymer production was essential for maintaining a stable state biofilm. Assuming that bioluminescence corresponds to alternate EPS induction, a model can be suggested in which exopolymer accumulation precedes biofilm sloughing, which allows other, possibly nutrient rich areas, to be colonized. In the case of NaCl induction, under the same assumption, exopolymer accumulation may be essential in order for biofilm bacteria to become salt tolerant, thereby restoring a more normal mode of biofilm growth. These studies illustrate the versatility and potential usefulness of this bioluminescent reporter plasmid for studying the roles of EPS in biofilm process and the effects of other environmental insults on these microbial biofilms. Future experiments should allow the cellular processes involved in microbially induced corrosion to be successfully studied from a molecular point of view.

Publications:

Rice, J.F. (1995) Use of a bioluminescent reporter to monitor exopolysaccharide production by environmental bacteria from corroded metal surfaces. Thesis, The University of Tennessee, Knoxville.

Rice, J. F., Fowler, R.F., Fleming, J.T., Arrage, A.A., White, D.C. and Sayler, G.S. (1995) Effects of external stimuli on environmental bacterial strains harboring an *alg*D-*lux* bioluminescent reporter plasmid for the study of corrosive biofilms. Journal of Industrial Microbiology 15:318-328

Wallace WH, Fleming JT, White DC, Sayler GS (1994) An *algD-lux* Bioluminescent Reporter Plasmid to Monitor Alginate Production in Biofilms. Microb Ecol 27:225-239

Wallace WH, Rice JF, White DC, Sayler GS (1994) Distribution of Alginate Genes in Bacterial Isolates from Corroded Metal Surfaces. Microb Ecol 27:213-223

Table 1. Environmental source of environmental isolates and the alginate genes similarities.

Bacteria	Source	*alg*D	*alg*G	*alg*-76	*alg*B
P. aeruginosa FRD1	Cystic fibrosis isolate	218[a]	170	55	143
P. aereofaciens G	Essential electrical cooling water	5	39	28	142
B. pumilus H	Reactor tubercle (interior)	6	19	4	0
P. putida I	Reactor vessel (solid debris)	187	1	0	54
P. putida L	Water storage tank	74	8	67	29
P. syringae M	Tubercle attached to weld	128	307	116	124
P. fluorescens N	Reactor Vessel (solid debris)	0	0	0	57
S. maltophilia O	Raw cooling water header (dark colored)	21	19	7	22
P. syringae P	Raw cooling water header (light colored)	38	111	2	43
A. veronii 310	Water pipe inlet nodules	20	0	0	7
P. aeruginosa 322	Pipe section with tubercles at site of weld	200	105	15	150
P. alcaligenes 324	Tubercles on carbon steel	123	53	22	17
P. alcaligenes 347	Pipe section (surface)	34	17	3	27

[a] Integrated optical densities obtained by scanning DNA slot blots with a Bioimage Visage 110 (data from Wallace et al. 1994b).

Table 2. Relative induction of bioluminescence by environmental stimuli.

Induction	FRD1	L23	M39	N15	O46
100mM NaCl	1.4[a]	2.1	10.9	1.8	6.8
200mM NaCl	1.5	3.3	25.9	2.2	29.6
300mM NaCl	2.0	3.2	55.4	2.7	107.0
6.75mM NH^4/3.25mM NO^3	1.3	1.3	0.6	0.9	0.7
3.25mM NH^4/6.75mM NO^3	1.9	1.3	1.5	1.4	1.2
10mM NO^3	2.7	2.6	1.0	4.5	0.8

[a] Bioluminescence Induction Factors (BIF) were computed by dividing the normalized peak bioluminescence by the appropriate control for each inducing condition.

Table 3. Relative induction of uronic acids synthesis in bacterial isolates.

Induction	FRD1	L23	M39	N15	O46
0mM NaCl	+[a]	++	ND[b]	++	ND
100mM NaCl	++	+++	ND	+ 1/2	ND
200mM NaCl	+++	++	ND	+ 1/2	ND
300mM NaCl	+++	+	ND	+	ND

[a] Qualitative comparison of accumulated uronic acids in culture supernatant during time course experiments. Results are not intended to be quantitative but rather represent the relative induction of uronic acids synthesis in bacterial strains. Uronic acids were assayed using the metahydroxybiphenyl/H_2SO_4/borate (MHB) assay as described in materials and methods.

[b] M39 and O46 demonstrated little reactivity to MHB colorimetric assays.

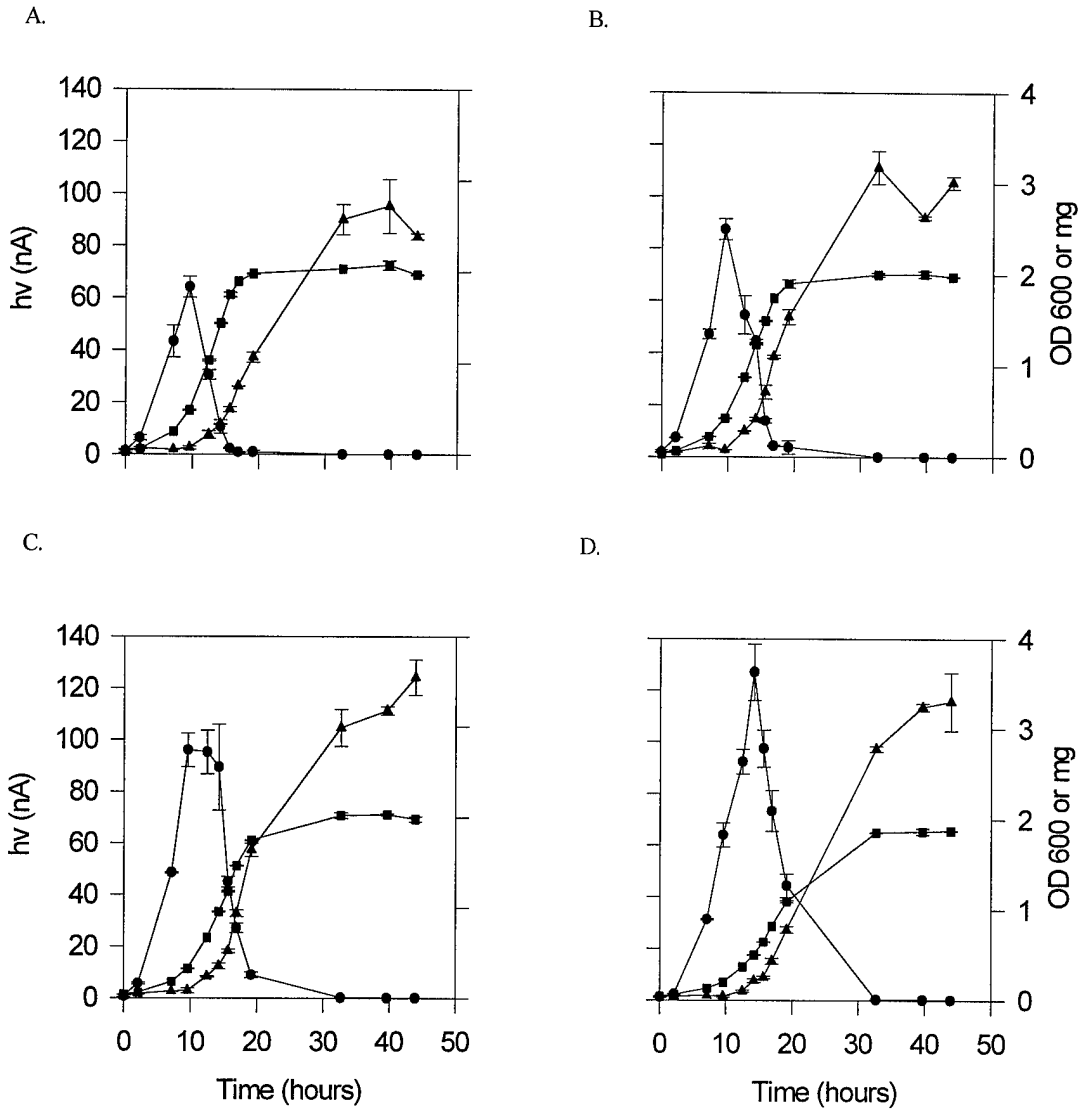

Figure 1. Bioluminescence readings (●), alginate accumulation (▲) and growth (■), during a time course experiment for *Pseudomonas aeruginosa* FRD1 grown in: A) APM; B) APM, 100mM NaCl; C) APM, 200mM NaCl; D) APM, 300mM NaCl. Bioluminescence was normalized by dividing the photomultiplier amperage by the absorbance of the culture at 600nm. Alginate was analyzed using the metahydroxybiphenyl/H_2SO_4/borate (MHB) assay as described in Material and Methods. Growth was monitored spectrophotometrically at 600nm. Each point represents the average from duplicate experiments, and error bars represent the standard error of this mean.

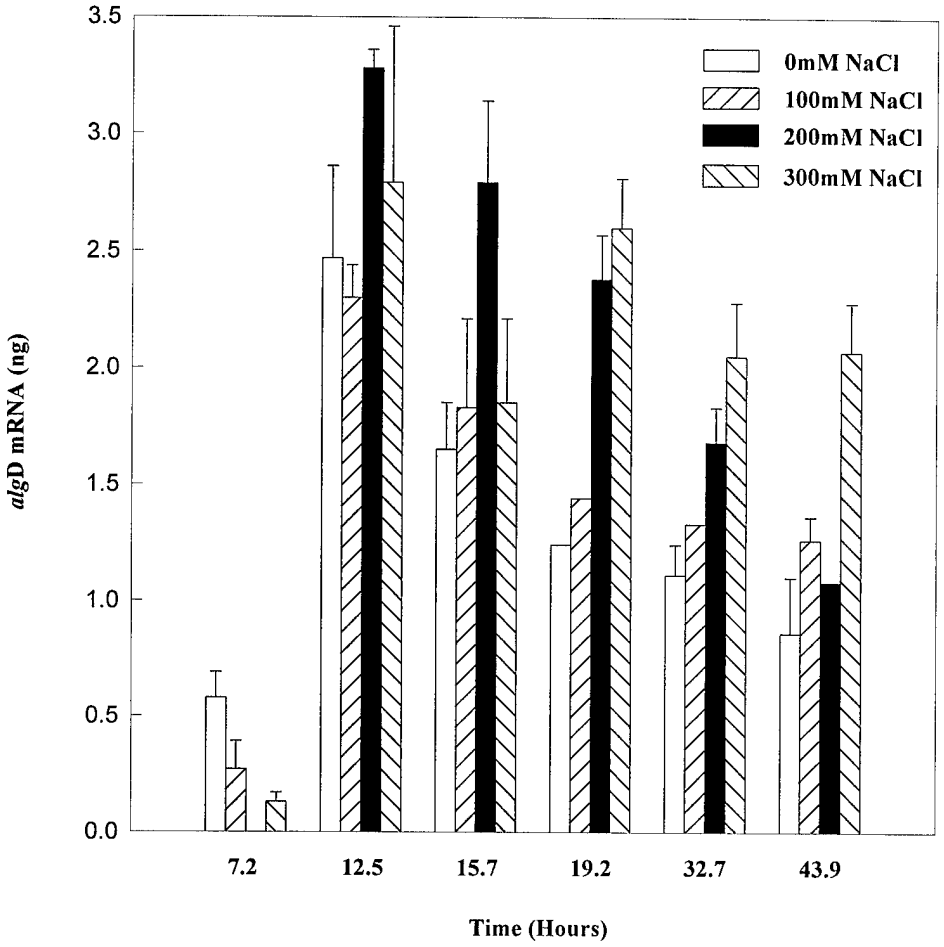

Figure 2. Abundance of *alg*D mRNA for *Pseudomonas aeruginosa* FRD1 grown in 50ml liquid cultures of APM (□); APM, 100mM NaCl (▨); APM, 200mM NaCl (■), APM, 300mM NaCl (▧). Values for mRNA were obtained from quantification of *alg*D mRNA slot blots using *alg*D sense strand RNA as a standard. Error bars represent the standard error of the mean of two samples.

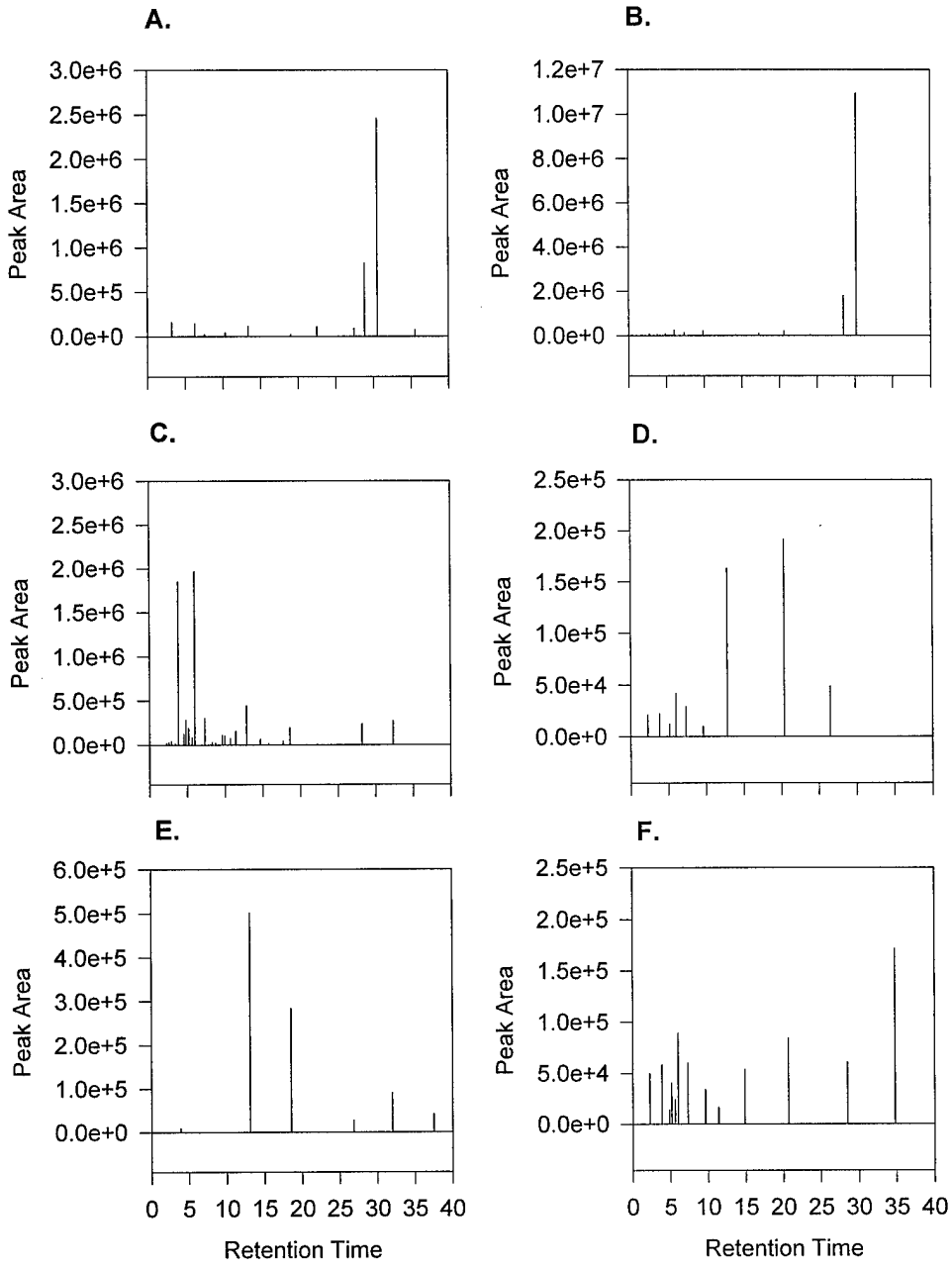

Figure 3. Ion Chromatography results using the Dionex Chromatography System as described in Materials and Methods: A) Alginate standard (1mg/ml); B) EPS (1mg/ml) from FRD1 grown in APM; C-F) EPS samples from environmental isolates (1mg/ml), L23, M39, N15, and O46, respectively, grown in APM.

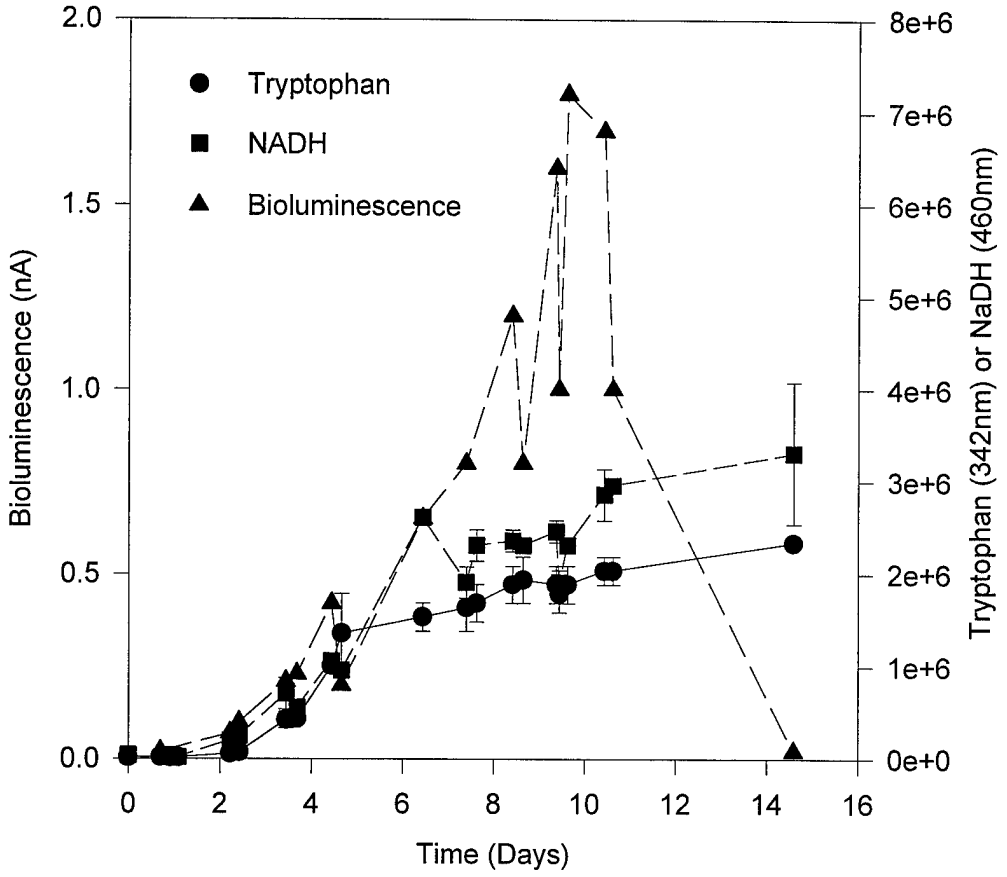

Figure 4. Bioluminescence and biomass determination in a biofilm containing *S. maltophilia* O46 using reactor A. Bioluminescence readings are expressed in nA (▲); fluorescence readings for tryptophan were taken at 342nm (●) and at 460nm for NADH estimates (■). Bioluminescence data represents the reading from the upstream port. Fluorescence readings were derived from averaging the readings from both flow cell ports. Error bars represent the standard error of this average.

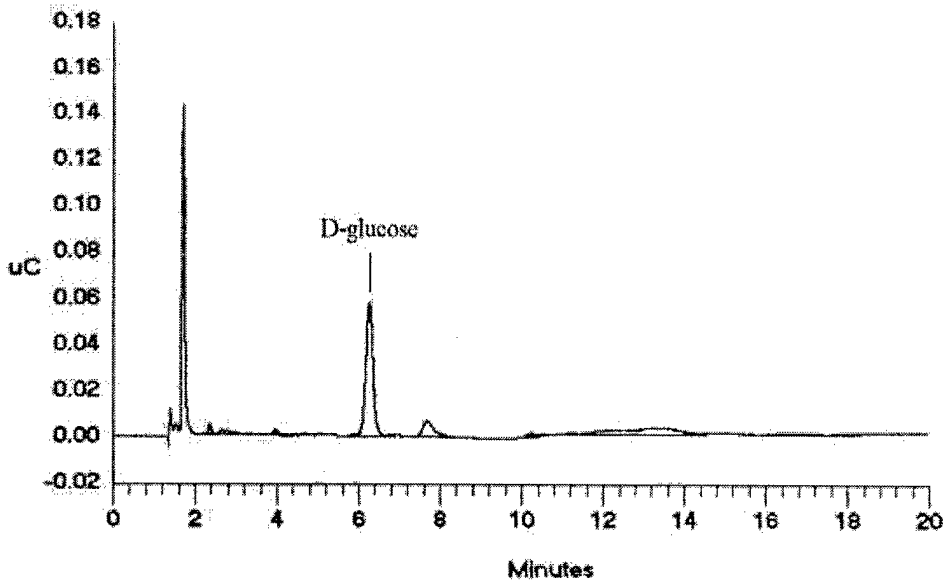

Figure 5. Dionex chromatograph of hydrolyzed exopolymer from a *S. maltophilia* O46 biofilms grown in Reactor B.

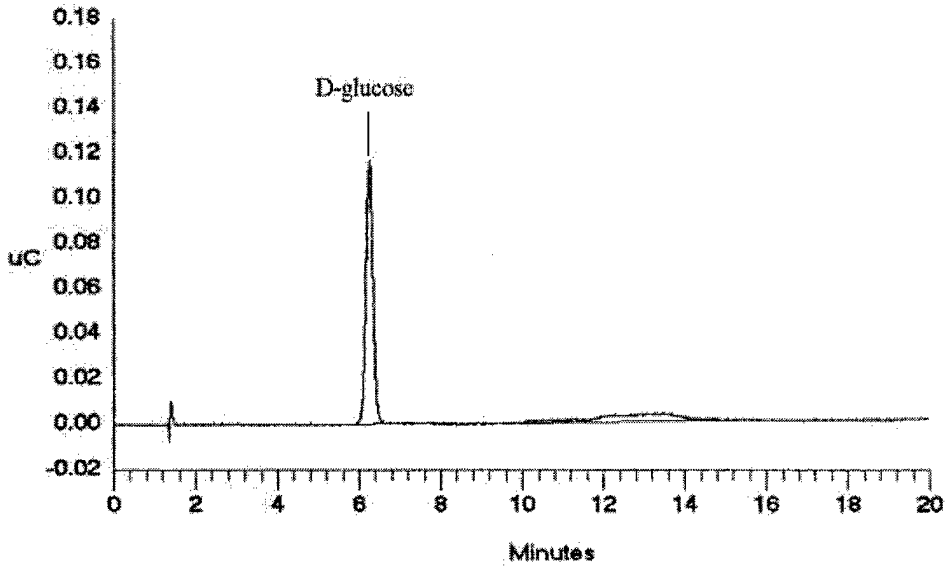

Figure 6. Dionex chromatograph of D-glucose.

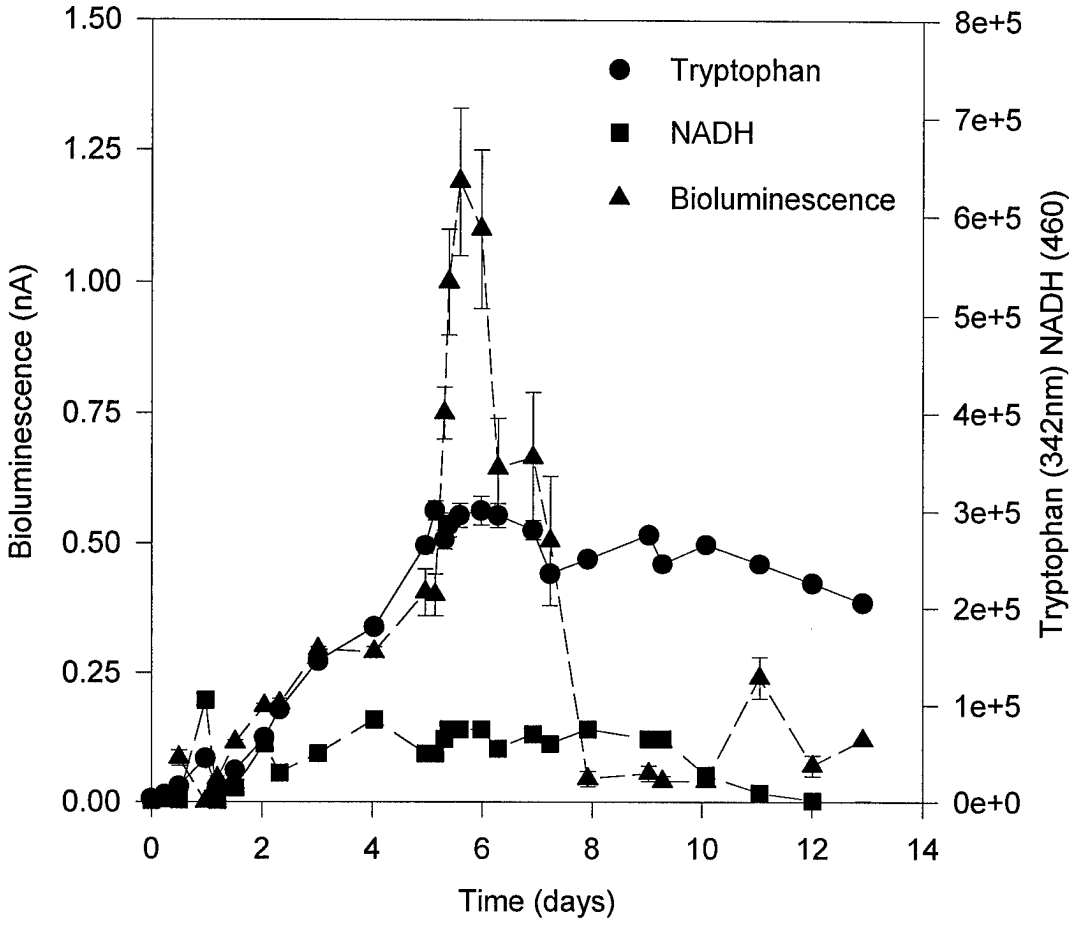

Figure 7. Bioluminescence and biomass determination in a biofilm containing *S. maltophilia* O46 using Reactor B. Bioluminescence readings are expressed in nA (▲); fluorescence readings for tryptophan were taken at 342nm (●) and at 460nm for NADH estimates (■). Bioluminescence and fluorescence data were derived from averaging two independent readings, one from the upstream side of the lens and one from the downstream side of the lens.. Error bars represent the standard error of this average.